Nodavirus encephalopathy in turbot (*Scophthalmus maximus*), Inflammation, Nitric oxide production and effect of antiinflammatory compounds.

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Summary

Nodaviruses are the ethiological agents of one of the most serious viral diseases affecting marine fish aquaculture. Nodavirus infection produces an abnormal swimming behaviour and causes encephalopathy and retinopathy associated to important mortalities. The expression of TNF-α, IRF-1 and Mx was up modulated in turbot after nodavirus infection. A significant increase in the production of nitrogen radicals was also observed in experimentally infected turbot. Several antiinflammatory compounds (the antioxidant N-acetilcysteine, cortisone, dexamethasone, prednisolone and aminoguanidine) were assayed to determine the role of inflammation on nodavirus infection. Cortisone and aminoguanidine were able to accelerate the mortality onset associated to nodavirus infection, modulating the gene expression of TNF-α and, in addition, modifying the arrival time of nodavirus to the brain. These results suggest the importance of early inflammatory processes to overcome the infection.

Introduction

Piscine nodavirus (family *Nodaviridae,* genus *Betanodavirus*) [1] are the causative agents of one of the most serious viral diseases that affects to marine fish larvae and juveniles in aquaculture [2]. The central nervous system (CNS) and the eye are the target sites for nodavirus replication. Affected fish exhibit swirling swimming and vacuolization and necrosis of the central nervous system. Turbot (*Scophthalmus maximus*), a marine fish species of growing economical importance in aquaculture, has been reported as being susceptible to several nodavirus strains isolated from striped jack [3] and Atlantic halibut [4]. Natural outbreaks have been described in Norway [5, 6] but the information on the immune response against nodavirus in turbot is scarce [7].

A strong expression of proinflammatory cytokines has been described in infected sea bass (*Dicentrarchus labrax*) brain. This response could be related with the neurodegeneration and encephalopathy associated to nodavirus infection since in seabream (*Sparus aurata*), a resistant species, the expression of these cytokines in infected animals was much lower [8]. In higher vertebrates, neuroinflammatory reactions associated to viral encephalitis caused by Herpes simplex virus type –1 (HSVE) involves a rapid production of IFN-γ and proinflammatory cytokines such as IL-1β, TNF-α [9, 10]. The effects of corticosteroids in controlling inflammation and certain forms of cerebral oedema have been experimentally documented on some viral encephalitis [11]. However, their role has been discussed and there is certain concern on whether their immunosuppressive action may result in an accelerated spread of the virus via the nervous system [12, 13].

The goal of our study was to study the nodavirosis in turbot and to determine the role of several antiinflammatory factors in turbot nodavirus pathogenesis.

Materials and methods

Animals

Turbot, of 2.5-5 g total weight, were obtained from a commercial fish farm (Insuiña, Acuinova Spain). Fish were acclimated to laboratory conditions for 2 weeks, maintained at 20º C and fed daily with a commercial diet.

Viruses

The nodavirus strain AH95NorA isolated from Atlantic halibut (*Hipoglossus hipoglossus*) included in BFNNV (*barfin flounder nervous necrosis virus*) betanodavirus genotype and the 475-9/99 strain from sea bass included in SJNNV (*striped jack nervous necrosis virus*) genotype were provided by the Institute for Marine Research (Bergen, Norway) and the Instituto Zooprofilattico Sperimentale delle Venezie (Italy) respectively. The viruses were propagated in the SNN-1 cell line as previously described [14], titrated in 96-well plates (Falcon, BD) and maintained with Minimum Eagle Medium (MEM) (GIBCO) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO) and 2% Foetal Calf Serum (FCS) (GIBCO). TCID50 ml^{-1} (tissue culture infectious dose infecting 50% of inoculated cultures) was calculated according to Reed and Münch [15].

Experimental infection with nodavirus

In order to confirm the virulence of the two nodavirus strains for turbot, groups of 22 animals (2,5 g mean total weight) were infected by intramuscular injection (i.m) with the AH95NorA or the 475-9/99 strains (100 μl/ fish, 1 x 10^6 $TCID_{50}$ ml^{-}). The control group was injected with culture medium. All groups were maintained in the same experimental conditions.

Histology

After 7 days pi, four nodavirus infected fish and four controls were sacrificed and samples of different organs were fixed in Davidson's fixative [16], processed in an automatic tissue processor (Reichert-Jung Histokinette 2000) and embedded in paraffin. Sections of five-micrometer in thickness were cut, deparaffinized, rehydrated and then stained with haematoxylin and eosin (H&E) for histopathological examination.

Isolation and maintenance of head kidney and blood leukocytes and brain cells.

Head kidney and blood leukocytes were isolated following the method previously described [17]. The viable cell concentration was determined by Trypan blue exclusion and adjusted to $2x\ 10^6$ cell ml^{-1}

In order to obtain a primary culture of brain cells, entire brains were removed in sterile conditions, placed into culture medium and dissociated by forcing it through a 100 μm nylon mesh. The suspension was centrifuged at 500xg for 15 min and the resulting precipitate was resuspended in Leibovitz medium (L15) supplemented with Foetal Calf Serum (FCS) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell concentration was adjusted to $1x\ 10^7$ cell ml^{-1}.

Nodavirus detection by RT-PCR.

In order to determine the presence of nodavirus in brain and kidney of infected turbot, viral detection was performed using RT-PCR based on the amplification of a highly conserved region of the coat protein gene as previously described [18]. Products of the amplification reaction were visualized on a 1.5% agarose gel.

Effect of nodavirus on nitric oxide (NO) production of turbot kidney leukocytes and brain primary cells

The production of nitric oxide (NO) was quantified using a method based in the Griess reaction that quantifies the nitrite content in cell culture supernatants [19].

To study the i*n vivo* effect of nodavirus in the production of NO of kidney leukocytes and brain cells isolated from *in vivo* infected fish, 12 turbot were i.m. injected with 100 μl of AH95NorA (1 x 10^6 $TCID_{50}$ ml^{-1}) and 12 fish were injected with 100 μl of cell culture medium as control. Fish were killed with an overdose of 2-Phenoxyethanol (Sigma, Aldrich) at 1, 3 and 7 days post infection and brain and head kidney were aseptically removed. Leukocytes from head kidney and brain primary cell cultures were obtained as described in the above section and adjusted to 2 x 10^6 cell ml^{-1} and 1 x 10^7 cell ml^{-1} respectively. The NO production was measured after 6h, 12h, 1, 3 and 7 days post sampling. The experiment was performed three times. The results are expressed as mean of all experiments conducted.

The *in vitro* NO production was tested after *in vitro* inoculated head kidney turbot leukocytes (2x10^6 cells ml^{-1}) with two doses of nodavirus (low dose 1x10^3 $TCID_{50}ml^{-1}$, high dose 1x10^6 $TCID_{50}ml^{-1}$). After 6h, 12h and 1 day post sampling, the NO production was measured in the supernatants.

The data are expressed as stimulation index that were obtained by dividing the infected cells values by those of control cells.

Cytokine induction after a nodavirus infection.

The level of expression of Mx, TNFα and IRF-1 was determined on tissues of experimentally infected turbot by Real Time RT-PCR (qPCR).

The *in vivo* induction was assayed on turbot experimentally infected by intramuscular injection. Sixteen fish were injected with 100 μl of nodavirus (1 x10^6 $TCID_{50}$ ml^{-1}) and another sixteen fish were used as controls and i.m. injected with 100 μl of cell culture medium. Fish were sampled at 4h, 1, 3 and 7 days post infection and the brain was aseptically removed and frozen for RNA isolation and cDNA transcription by Superscript Preamplification System (Gibco). Pools were made with four fish brains.

Quantitative PCRs were performed using the 7300 Real Time PCR System (Applied Biosystems). cDNA amplification was performed using specific primers

designed with Primer 3 software [20]. Each primer (0.5 µl of 10µM) was mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95º 15s and 60º for 1 min. The comparative CT method (2-ΔΔCT method) was used to determine the expression level of analysed genes [21]. The expression of the candidate genes was normalized using RNA 18S ribosomal gene turbot as a housekeeping gene, after confirming that there was not modulation of this gene with the infection. Fold units were calculated dividing the normalized expression values of infected tissues by the normalized expression values of the controls. Primers sequences are shown in table 1.

Effect of administration of different modulators of inflammation in the mortality caused by nodavirus infection.

With the aim to determine if the inflammatory response of nodavirus infected turbot could play a significant role on the disease pathogenesis, several antiinflammatory compounds were administered together with the virus. Groups of twenty turbot were injected intramuscularly with 100 µl of nodavirus AH95NorA strain ($1x10^6$ $TCID_{50}ml^{-1}$) for each treatment. Controls received the same volume of culture medium (MEM plus 2% FCS). After these injections, several treatments were administered as follows:

- The antioxidant N-acetilcysteine (NAC, Sigma) (3 mg/ fish) was intraperitoneally injected after nodavirus or medium inoculation and administered daily until the end of experiment. The chosen doses were used following previous studies on zebrafish [22].
- After the infection procedure, groups of 20 fish were injected i.p daily until the end of experiment with 100 µl of cortisone (10µM), Dexamethasone (50µg/ml) or Prednisolone (750µg/ml). In all cases, doses were adjusted to fish weight as described in the literature [23, 24].

- One hundred of Aminoguanidine hemisulfate salt (Sigma Aldrich, Co), (0,5 mg/kg of fish) [25] was intraperitoneally injected, after nodavirus infection, daily until the end of experiment.

Fish control groups treated only with NAC, cortisone, dexamethasone, prednisolone and aminoguanidine were maintained and injected daily with the same dose than infected fish. Moreover, a nodavirus group was injected i.p. daily with PBS. Experimental infections were performed two or three times.

Effect of cortisone and aminoguanidine on nodavirus detection and gene expression.

Groups of twelve turbot were i.m. injected in the conditions described above with either 100 µl AH95NorA nodavirus (1 x 10^6 $TCID_{50}$ ml^{-1}), 100 µl of nodavirus and a daily dose of cortisone, only a daily dose of cortisone, 100 µl of nodavirus and a daily dose of 100 µl of aminoguanidine or only a daily dose of 100 µl of aminoguanidine. Control group was injected with 100 µl of cell culture medium.

After 1, 3 and 7 days post infection brains were removed and RNA obtained (tissues were pooled from 4 fish for each treatment). Five µg were transcribed to cDNA as previously described in order to determine the expression of TNF-α as described above. Other 5 µg were used to determine the nodavirus presence by RT-PCR as described above.

Data analysis

Data were compared using the Student's *t*-test. Results are expressed as mean ± standard deviation and differences were considered statistically significant at $p < 0.05$. Analysis of variance (ANOVA) was used for the comparison of mortality rates from each treatment and significant differences were evaluated using the Tukey multiple comparisons post hoc test using SPSS software.

Results

Mortality rates and replication of nodavirus in turbot

Both nodavirus strains caused mortalities in i.m. injected turbot and caused dark pigmentation and "swirling" swimming behaviour. Both betanodavirus strains (475-9/99 and AH95NorA), produced high mortality rates when injected at the same viral titre, 1x 10^6 $TCID_{50}ml^{-1}$ (Figure 1A). In the case of 475-9/99 the mortality began before, at 7 days p.i.

Moreover, after 1 and 3 days p.i., nodavirus were detected at low level in the brain. Its presence was higher at 7 day p.i (Figure 1B). Nodavirus was not found in head kidney at the different sampled points (data no shown).

Histopathology

A histological examination was conducted in brain, kidney and retina from turbot. After seven days postinfection, the symptoms were similar to those detected in other susceptible species (Figure 2). A strong vacuolization was observed in brain and retina (Figure 2B and D) between the granular and bipolar layers. Haemorrhages in interstitial tissue and elimination of connective tissue were observed around the renal tubules in kidney (Figure 2F).

NO production after a nodavirus infection in brain cells and head kidney leukocytes.

The ability of head kidney leukocytes and brain cells to produce NO was examined in response to nodavirus infection. More than 60% of the examined infected fish showed a significant increase in NO production (Table 2). In all sampled points the nitrite concentration was higher in infected than control fish. *In vitro*, the two doses of

nodavirus ($1x10^3$ $TCID_{50}$ ml^{-1} and $1x10^6$ $TCID_{50}$ ml^{-1} respectively) assayed showed a nitrite concentration increase at 1 day post infection (Figure 3) in head kidney leukocytes. In brain cells, NO was not detectable *in vitro* (data not shown).

Cytokines expression analysis in turbot after a nodavirus infection

The expression levels of some proinflammatory cytokines such as TNF-α, IRF-1 and antiviral cytokines such as Mx determined by quantitative PCR are shown in Figure 4. The expression of Mx was increased at 3 and 7 days post infection being higher at 7 days p.i. (Figure 4A).

A significant up-modulation of IRF-1 and TNF-α expression was detected in the brain at 3 day p.i (Figure 4B and 4C, respectivelly). At 7 days p.i. the expression continued to be higher than in the uninfected group but the expression level decreased.

Effect of inflammation modulators in mortality rate associated to nodavirus infection.

The daily treatment of infected fish with NAC resulted in a 20% reduction of the cumulative mortality (Figure 5A). However, the difference between the treated and untreated group was not statistically significant.

The effects of a daily treatment with the immunosupressors cortisone, dexamethasone and prednisolone are shown in Figure 5 B, C and D. All of them induced earlier mortalities in nodavirus infected turbot. Mortality percentage in the infected and daily cortisone treated fish group began as early as 4 days p.i, reaching 90 % of cumulative mortality (day 22 p.i). Mortality of infected and treated group was always higher than that of the infected but not treated group (Tukey test, $p= 0.0397$, $p<0.05$).

Although the treatment of nodavirus infected fish with dexamethasone and prednisolone (Fig. 5C and D) produced mortality similar to that caused by the virus, both treatments produced an acceleration at the mortality onset in early times post infection.

In the group of nodavirus infected turbot and daily treated with aminoguanidine (Figure 5 E), mortality increased gradually and was significantly higher (p= 0,001) than that of infected and untreated fish.

Effect of cortisone and aminoguanidine in the viral load and in the expression of inflammatory cytokines.

The presence of nodavirus was detected in the brain of infected-untreated fish at 3 day p.i. but not before. However, when cortisone or aminoguanidine were administered, an earlier and higher level of nodavirus detection was observed as soon as 1 day p.i. in brain (Figure 6A).

A significant down-modulation of the expression level of TNF-α, was detected in the brain of infected and cortisone or aminoguanidine treated fish (Figure 6 B and C) compared with the fish infected only with nodavirus. This down-modulation was transitory because at 7 days p.i. there was an up-modulation of the expression level in the treated and infected group compared to those of infected and not infected groups.

Discussion

Although in higher vertebrates inflammation in CNS has been reported to be an important mediator in pathogenesis [26], in fish, the correlation between the increase of of proinflammatory cytokines expression and CNS lesions is, according with previous studies, not evident [27].

In sea bass and sea bream the response against nodavirus is characterized by NO production and by an up modulation of the expression of Mx, TNF-α and IRF-1. NO may have an antiviral effect in response to virus [19, 28], however, there are controversial reports in mammals [29, 30]. This is the first study that describes increased NO production in fish brain cells in *in vivo* infected fish by a nodavirus. It was not possible to detect NO production in brain cells *in vitro*, probably due to the lack of knowledge on how to maintain these cells for longer periods.

TNF-α is an important mediator of proinflammatory functions in human viral diseases [31, 32], and it has been proposed as the most important molecule involved in the vacuolization of neuroinflammatory processes associated to human cerebral diseases [33, 34, 35, 36, 37]. IRF-1 (interferon regulatory factor 1) activates genes associated to antigen presentation and it is induced by gamma interferon and Mx is one of the best known effector molecules of interferon α/β [38, 39]

As it has been reported for sea bream and sea bass infected with nodavirus [8], we describe an up modulation of TNF-α in infected fish brain.

The expression level of IRF-1 gene showed a transitory up-modulation at day 3 post-infection which did not last after 7 days p.i. Little is known about the activity of the interferon genes in teleost fish but the antiviral defence, immune regulation and cell growth can be counted among the physiological roles of IRF-1 [40, 41]. In fact, very recently, a study was reported in flounder showing evidences of antiviral ability to IRF-1 against virus [42].

As described in several mammalian species [43, 44, 45, 46], Mx might contribute to non specific resistance against viruses. In fish, the antiviral activity of Mx has been previously described *in vitro* in several species such as the Japanese carp *(Carassius auratus)* [47], Atlantic salmon [48] and grouper [49] and recently, barramundi *(Lates calcicifer)* [50] reporting that the viral activity is virus-specific being higher against nodavirus than to iridovirus. Nodavirus induces the expression of Mx mRNA in brain, and this up-modulation exhibits a similar expression pattern than viral RNA load in this organ being higher at 7 days post infection. Moreover, a high

modulation of Mx in nodavirus infected brain was reported in grouper [51] which interacts with coat protein of virus and sea bream [8]. These results suggest an antiviral role of Mx that could be induced by the virus and not by the inflammatory cascade in the brain according to other studies [51]. The involvement of Mx and the interferon system has been recently reported in fish infected with nodavirus [52, 53, 54].

The inflammatory response is an indispensable defence mechanism against pathogens, but it may also damage tissues. In fact, a dual role of inflammatory response in the brain has been reported after a CNS injury, a response that can aggravate the damage in affected CNS but that can also participate in neural regeneration [55].

The corticosteroids are well known for their anti-inflammatory and immunosuppressive properties [56]. In our study, the daily administration of cortisone seems to accelerate the mortality onset. The same results were observed when dexamethasone and prednisolone were administered, although these drugs produced an acceleration of the onset of the mortality but not an increase in the cumulative mortality. When the treatment with cortisone was delayed 7 days post virus inoculation (when fish began to have external signs), the mortality rate began at similar time than the untreated group (data not shown). This suggests the importance of an early inflammatory response in nodavirus infection.

In addition, corticosteroids may have an effect on the entrance and viral spread in the brain since they are immunosuppresors which can inhibit the brain primary response against the infection. In our experiments, the reduction of inflammation seems to produce faster mortalities. TNF, one of the main proinflammatory molecules, is known to activate immune cells such as microglia and to have a protective role for the brain in the first stages of the infection. These results have been also detected in rhabdovirus infection [57] and in general, in higher vertebrates it is well established a potent antiviral activity against a variety of virus. This contrasts with that reported by Roca et al. [58] probably due to that an excess of TNF can be also be detrimental for the host.

Since TNF-α and IFNs have the ability to induce iNOS expression in mammals system [59], it is possible that the AG treatment may affect the iNOS induction pathway, needed to fight the virus. Again, as it has been reported in mice infected with primary ocular Herpes simplex Virus [25], the NO production seems to have a neuroprotective role.

In summary, in a similar way as mammals, viral encephalopathy, in turbot nodavirus infection is characterized by a strong and transitory expression of proinflammatory cytokines in the brain. The different response between brain and head kidney could be explained because the brain is the target organ for the nodavirus infection, and in head kidney no viral replication has been detected. Moreover, this response seems to be essential at the early times of infection since an immunosuppressive treatment can alter the parameters associated with nodavirus infection and produce a modulation in the mortalities.

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545

Figure Legends.

Figure 1. Cumulative Mortality (%) of turbot infected i.m with two nodavirus strains, AH95NorA, from Atlantic halibut, or 475-9/99 from sea bass (10^6 $TCID_{50}ml^{-1}$) (n=22 in each group). B) Nodavirus presence was detected by RT-PCR in brain from turbot experimentally infected with the AH95NorA strain (10^6 $TCID_{50}$ ml^{-1}). The detection was conducted in two pools of two fish in each sampled point.

Figure 2. Light microscopy of tissue sections of brain, retina and kidney from experimentally infected turbot after 7 days pi, showing the damage induced by nodavirus (AH95NorA strain, 10^6 $TCID_{50}ml^{-1}$). Section of control (A) and infected (B) brain showing the vacuolization induced by nodavirus. The arrow indicates a zone with multiples vacuoles in brain cortex. C, control and D) infected retina, showing an extensive vacuolization indicated by an arrow between bipolar and granular layers. Kidney from control (E) and infected turbot (F) showing the presence of haemorrhages (arrow). Peritubular oedema and disruption in connective tissue are found around of renal tubules (asterisk).

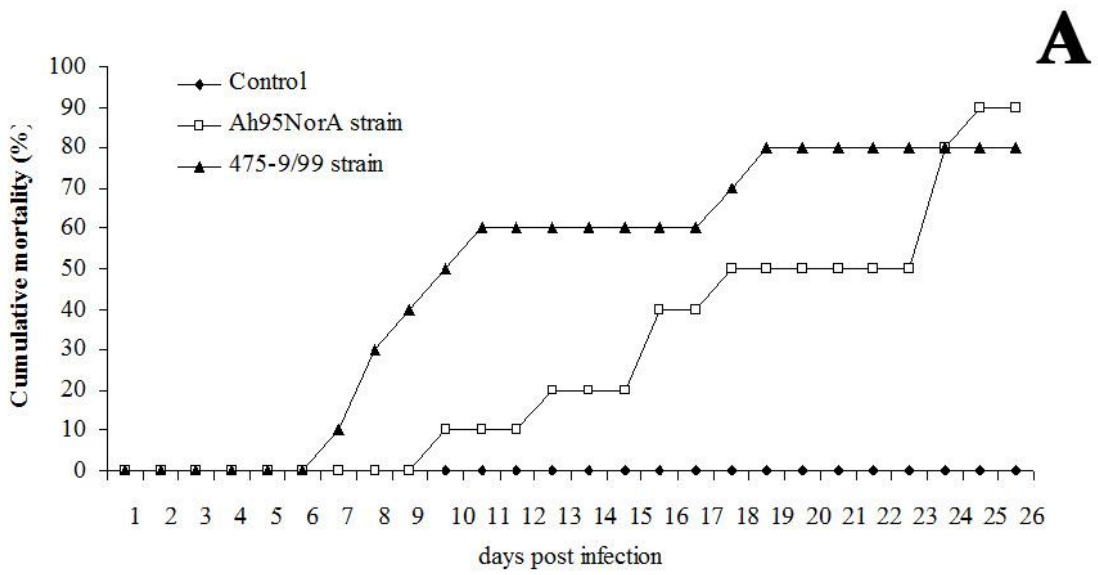
Figure 3. NO production from kidney leukocytes *in vitro* in response to a low dose (AH95NorA, 10^3 $TCID_{50}ml^{-1}$) and to a high dose (10^6 $TCID_{50}ml^{-1}$) of nodavirus. Data are represented as stimulation indexes compared with uninfected controls.

Figure 4. Real time RT-PCR for Mx (A), IRF-1 (B) and TNF-α C) relative transcription level in the brain of intramuscular infected turbot. Fold units were calculated dividing the expression values of infected tissues by the expression values of the controls once normalized regarding to rRNA 18s. The data represent mean of 4 pools for each treatment.

Figure 5. Cumulative mortality of the infected fish (AH95NorA strain 10^6 $TCID_{50}ml^{-1}$) treated or not with NAC (3 mg/fish) B) cortisone 10μM, C), dexamethasone (DEX) (50μg/ml) D), prednisolone (pred) (750μg/ml) E and Aminoguanidine (AG) (1 mg/ml) (F). The mortalities produced for each treatment in comparison to nodavirus infection were significantly different according to Tukey test (POST HOC) for cortisone and AG treatment (p=0,0397, p=0,00, respectively) $p<0.05$.

Figure 6. A. Nodavirus detection by RT-PCR in brain from turbot infected with nodavirus and treated with cortisone (Cort) (10 μM) or Aminoguanidine (AG) at different times post-infection. Each lane represents one pool (n= 4 fish) for each treatment. Lane 1: control; lane 2: nodavirus; lane 3: nodavirus-cortisone; lane 4: nodavirus-AG. B) Real time RT-PCR results for TNF-α relative transcription level in the brain of experimentally infected turbot and treated with cortisone daily. C) TNF-α expression level in the brain of experimentally infected turbot and treated with aminoguanidine daily. Fold units were calculated dividing the expression values of infected tissues by the expression values of the controls once normalized regarding to rRNA 18s. The data represent the expression of pools (n=4) for each treatment.

Figure 1.


A
Control
Ah95NorA strain
475-9/99 strain
Cumulative mortality (%)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
days post infection

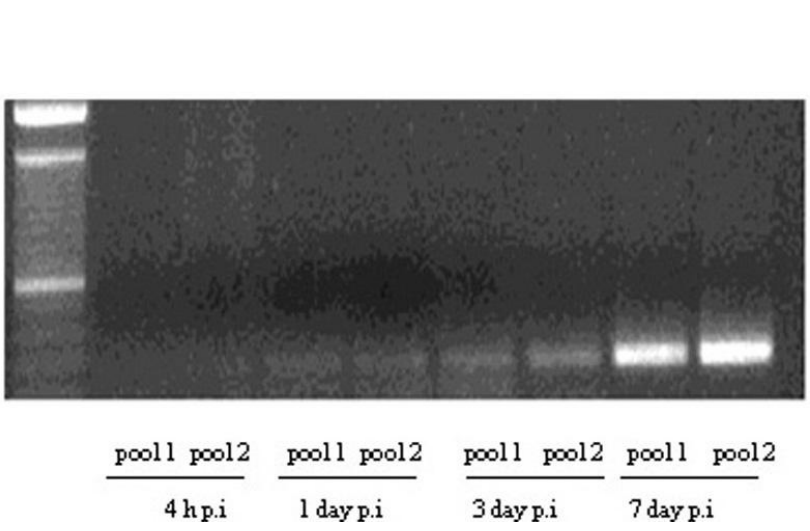

B
pool1 pool2
pool1 pool2
pool1 pool2
pool1 pool2
4 h p.i
1 day p.i
3 day p.i
7 day p.i

Figure 2.

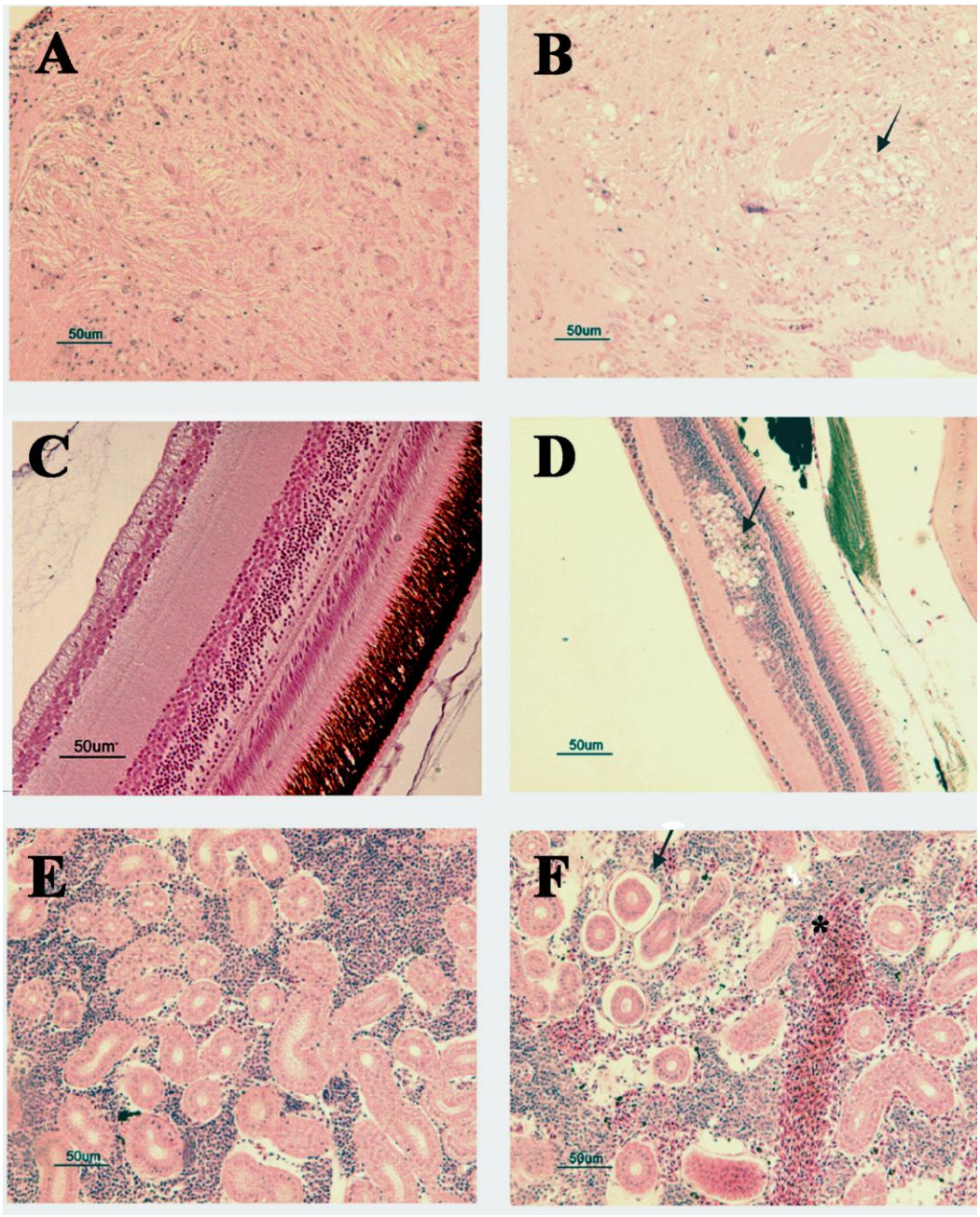


Figure 3.

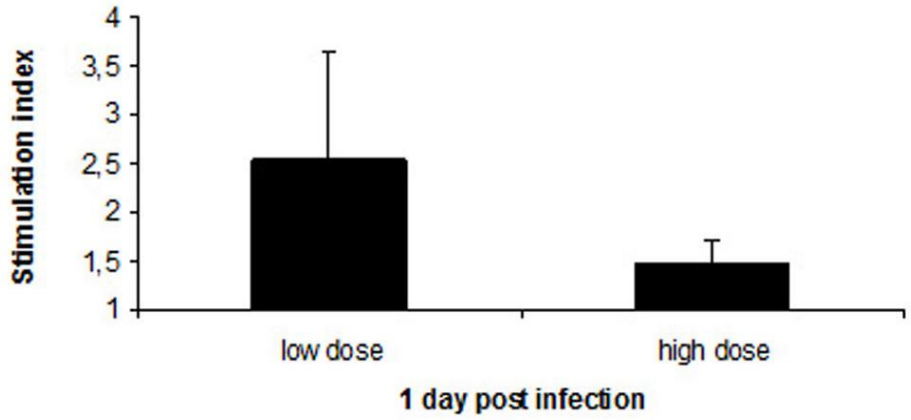

Stimulation index
4
3,5
3
2,5
2
1,5
1
low dose
high dose
1 day post infection

Figure 4.

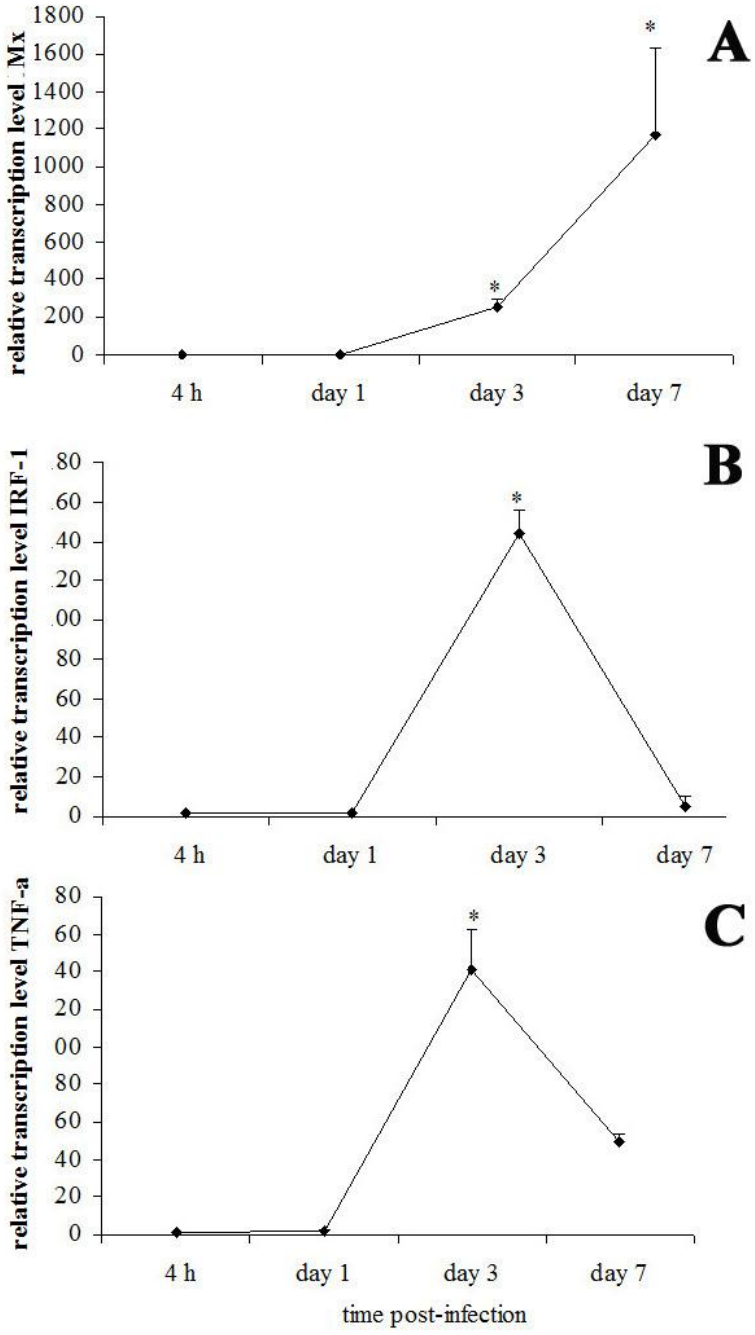

A
relative transcription level Mx
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
4 h
day 1
day 3
day 7
B
relative transcription level IRF-1
80
60
40
20
00
80
60
40
20
0
*
4 h
day 1
day 3
day 7
C
relative transcription level TNF-a
80
60
40
20
00
80
60
40
20
0
*
4 h
day 1
day 3
day 7
time post-infection

Figure 5.

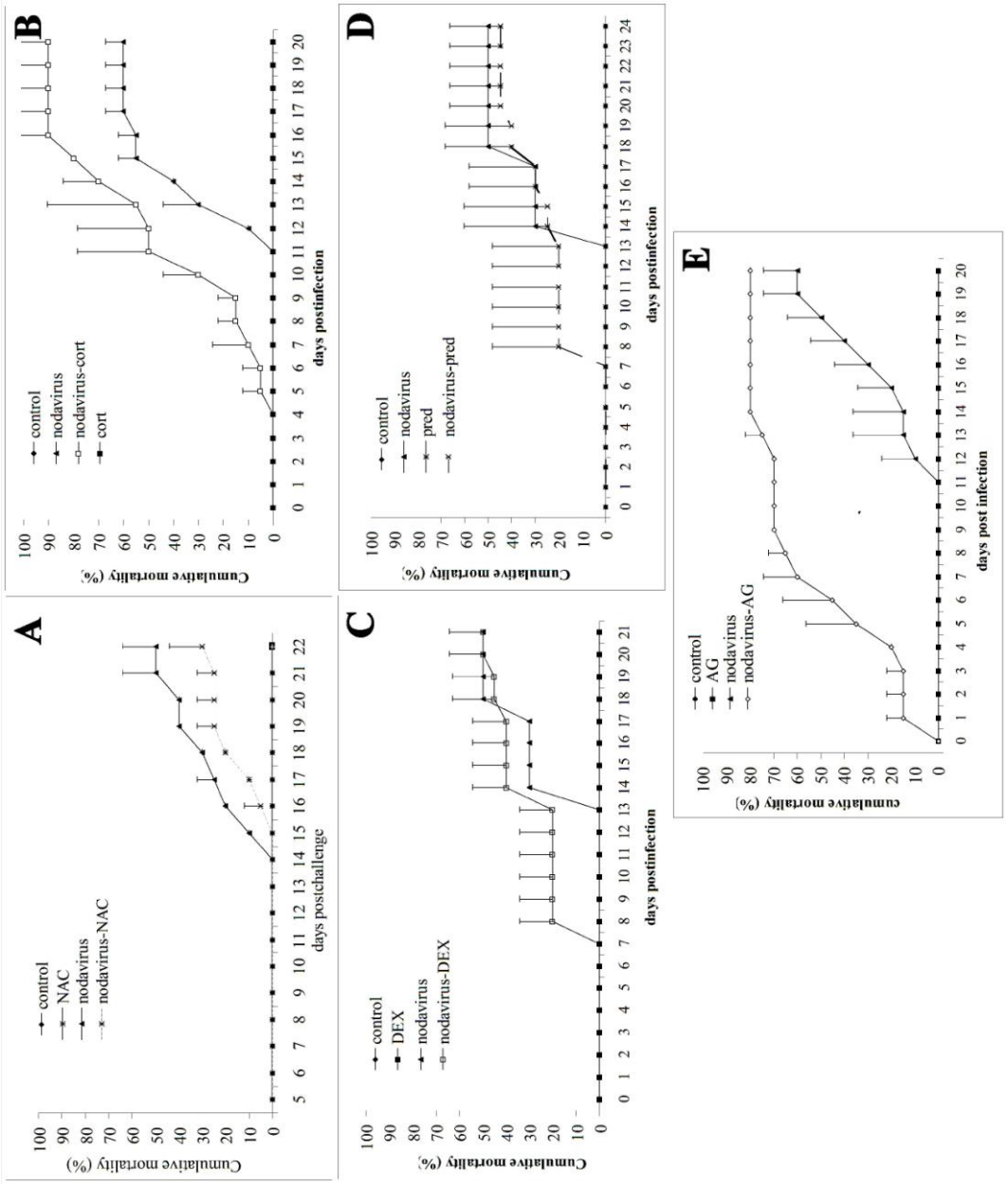

A
control
NAC
nodavirus
nodavirus-NAC
Cumulative mortality (%)
days postchallenge
B
control
nodavirus
nodavirus-cort
cort
Cumulative mortality (%)
days postinfection
C
control
DEX
nodavirus
nodavirus-DEX
Cumulative mortality (%)
days postinfection
D
control
nodavirus
pred
nodavirus-pred
Cumulative mortality (%)
days postinfection
E
control
AG
nodavirus
nodavirus-AG
cumulative mortality (%)
days post infection

Figure 6.

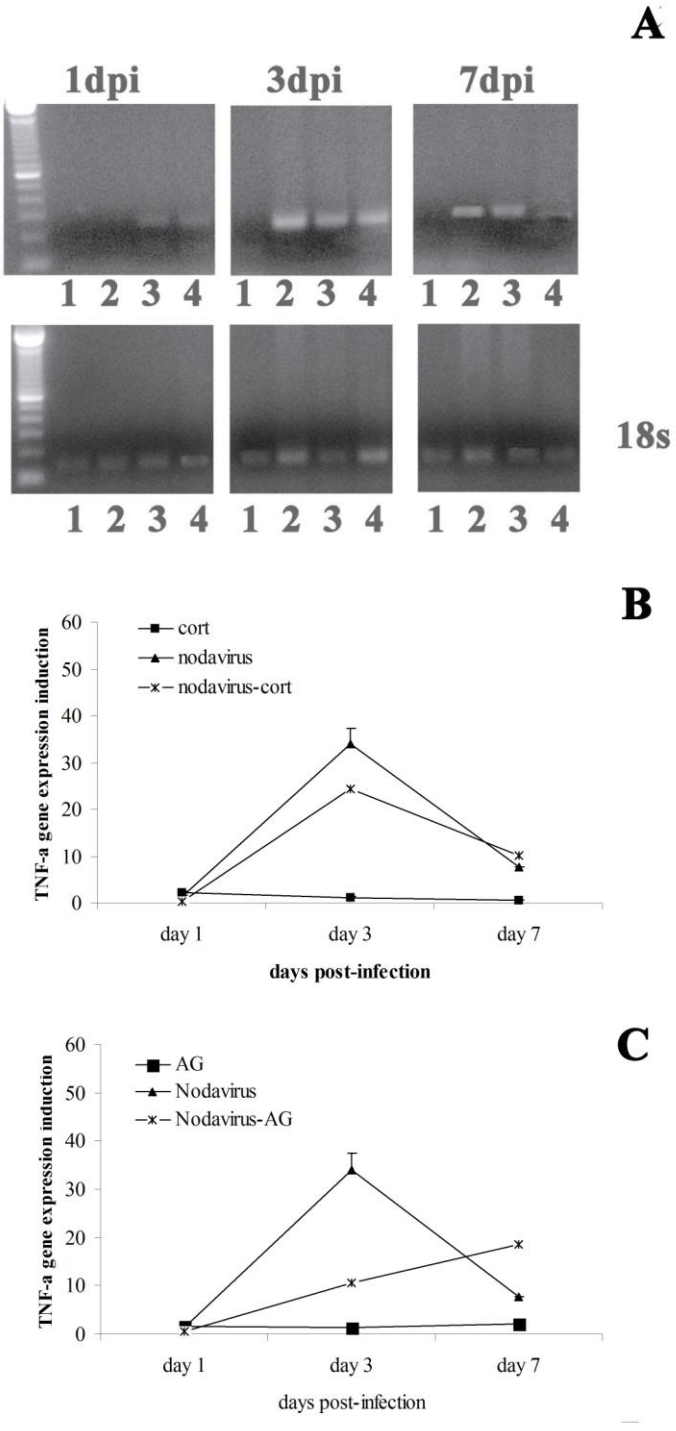

A
1dpi
3dpi
7dpi
1 2 3 4 1 2 3 4 1 2 3 4
18s
1 2 3 4 1 2 3 4 1 2 3 4
B
TNF-a gene expression induction
cort
nodavirus
nodavirus-cort
60
50
40
30
20
10
0
day 1
day 3
day 7
days post-infection
C
TNF-a gene expression induction
AG
Nodavirus
Nodavirus-AG
60
50
40
30
20
10
0
day 1
day 3
day 7
days post-infection

TABLE 1. Primers sequence of the genes analysed.

Gen	Primer sequence 5´-3´ Forward and reverse	Access no. (genbank)
rRNA 18S turbot	5`-ATGGCCGTTCTTAGTTGGTG-3´ 5´-CTCAATCTCGTGTGGCTGAA-3	EF126038
Tumor necrosis factor-α	5´-AGAGATGAGGACGCAGTCAAG-3´ 5´-TTGACCGTTCTTCCACTCCAG-3´	Ordás et al., 2007
Interferon regulator factor 1 (IRF-1)	5´-CTGACATCGAGGAGGT-3´ 5´-ATGCGCACGGCTTGGT-3	AY962253
Mx protein	5´-AGAGTTTGGGAAGTGGAGCA-3´ 5´-AGTTGATGAAGCCTGGCAGT-3´	AF245514

TABLE 2. Percentage of infected fish with significantly higher NO production than controls (stimulation index >1).

Percentage of infected fish with higher NO production compared than controls

	Days post infection				
	6 h	12 h	1	3	7
Head Kidney	*60*	*75*	*75*	*61,5*	*60*
Brain	*-*	*0*	*75*	*62,5*	*50*